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A RAPID METHOD FOR THE STUDY OF GENETICS IN LARGE POPULATIONS

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(With Six Text-figures)

One of the important obstacles to the understanding of the mechanism of the origin of neoplasms has been the impossibility of studying the proliferation of single tissue cells in large numbers. Were that possible, even under conditions less favourable than those existing in the body, variation in cell proliferation with tumour formation could be detected if it occurred and the conditions of its occurrence and development studied. The difficulty of attacking this problem directly suggests an approach by analogy—studying rapidly multiplying bacteria.

The principal difficulties usually encountered in the study of the genetics of rapidly multiplying unicellular organisms have been the following:

(1) The minuteness of these organisms makes many of their morphological characteristics invisible, or if visible, indistinctly so.

(2) Those characteristics such as size, shape, capsule, staining qualities, etc., which can be distinctly observed, change so markedly with environmental conditions that genetic variations can hardly be distinguished from developmental ones(1).

(3) Even if definite morphological criteria could be established, the necessity for isolating the individual cells observed would make the entire procedure so cumbersome that the advantages of rapid multiplication would be entirely lost.

In order to avoid these difficulties we have studied macroscopic characteristics of cultures instead of microscopic characteristics of cells, characterizing the cell in terms of the type of colony grown from it under standard conditions.

An "unstable" culture of *Salmonella Aertrycke* was studied(2). This culture, during the exponential phase of growth, constantly gave rise to cells that grew into colonies grossly different from the parent type. Under continuous observation since December 1933, whenever plated out it gave rise to two types of colonies, one a large, low, rough colony

and the other a small, dome-shaped, smooth colony (Fig. 1). The rough colony, when plated out, always gave rise to both types; but the smooth colony always gave rise to smooth colonies only.

Since a rough colony when plated out gives rise to two types of colonies on the same plate under the same conditions, there must be at least two types of cells in the colony. But since a smooth colony gives rise only to smooth colonies, a smooth cell may be *defined* as one which

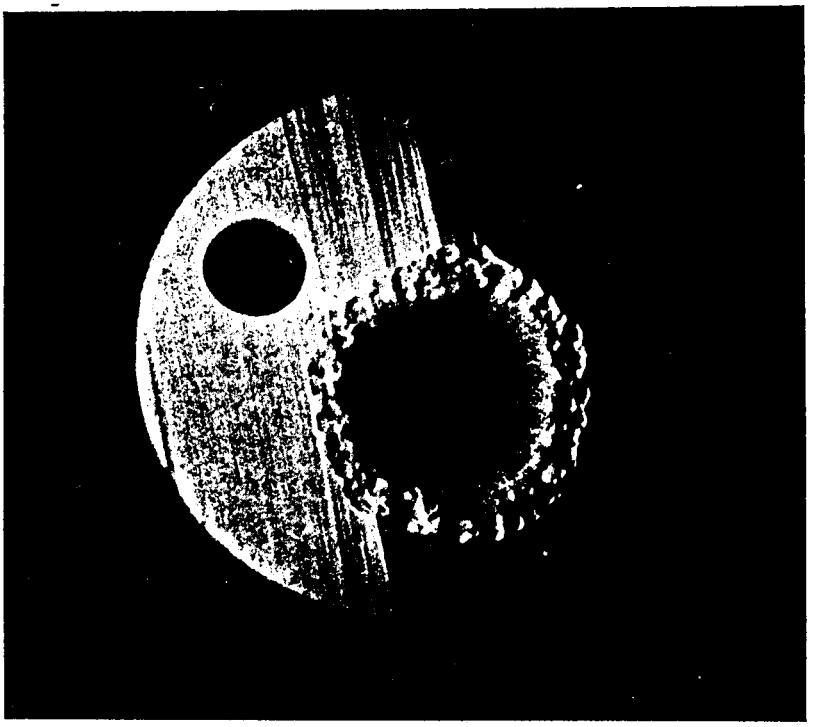

Fig. 1. Photograph of rough (right) and smooth (left) colonies of *Salmonella Aertrycke*. M.T. 2 C.

by itself, on a standard agar plate, can give rise only to a smooth colony. A rough cell may be *defined* as a cell which, by itself or with one or more smooth cells, can give rise to a rough colony. Since, however, in the thousands of rough colonies that have been examined smooth cells have always occurred, we are forced to conclude either that a rough cell as such can never exist independently of one or more smooth cells, or that rough cells constantly give rise to smooth cells which are in the nature of genetic *mutants*.

To settle this question, a broth suspension of 100 million organisms per c.c. (estimated by turbidity) was made from a rough colony and, by the micro-isolation technique described by Kahn (3), sixteen single-cell isolations were made and inoculated on the surface of agar plates. Six of these grew into colonies, of which four were rough and two were smooth. On subsequent plating the smooths gave rise to smooths only, while each rough gave rise to both rough and smooth colonies. Apparently, of the six cells which grew into colonies, four were rough cells and two were smooth.

Since microscopically all six were of so nearly the same dimensions as to be indistinguishable, it is manifest that the single rough cells could not have been accompanied by undetected smooth cells. We can therefore disregard the possibility that a rough cell cannot exist independently of one or more smooth cells, and conclude that the development of smooth cells from the parent rough cells is true genetic mutation.[1]

TECHNIQUE

(1) *Media*

All of the studies here described were made on plates of Bacto-Beef heart infusion agar, autoclaved for 20 min. at 18 lb. steam pressure in 170 c.c. batches. The agar was melted in boiling water, 1 day before use, and brought to a boil with a micro burner, to dissolve the sediment formed on autoclaving and to prevent the formation of inhomogeneities in the plate on cooling. It was then cooled to about 50° C. and poured into 100 mm. Petri dishes, about 17 c.c. to each plate. The plates were left in the incubator for 24 hours at 37° C. and, if sterile, used immediately thereafter.

(2) *Plating out*

A portion of the colony to be plated is picked up on a flamed loop previously cooled in sterile agar and streaked out on the plate as shown in the diagram (Fig. 2). No plate was used unless it contained at least 150 well-separated colonies.

[1] An alternative explanation of these findings would be, that a rough cell is merely a smooth cell contaminated by an ultra-microscopic virus; and that those of the progeny of the rough cells which continue to be so contaminated give rise to rough colonies, while those which are not so contaminated give rise to smooth colonies. There is, however, no evidence whatsoever for the existence of such a virus. Further, since the same type of *a priori* reasoning might equally well be extended to include the assumption of ultra-microscopic contamination of chromosomes as the source of genetic mutation, we feel safe in disregarding it.

(3) *Differential counts*

Counting was done with two Veeder counters, one held in each hand. The plate was examined upside down by transmitted light under the microscope with a magnification of 56 diameters. Rough colonies were counted by the right hand and smooth ones by the left. At least 100 colonies were counted on each plate, usually about 150. Each plate was counted three times and the results averaged.

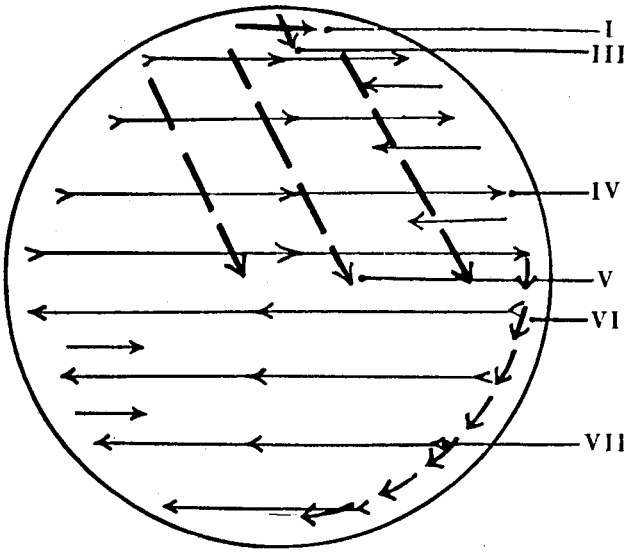

Fig. 2. Method of inoculating plates for differential counting. I. Loop containing sample of culture to be plated is rubbed on the edge of the plate. II. The loop is flamed and cooled in sterile agar. III. The cooled loop is touched to the heavily inoculated portion of the plate and lifted away. IV. The loop is then streaked back and forth across the plate rapidly, up to the middle of the plate. V. The loop is drawn down vertically across the streaks three times. VI. The plate is turned through 180°. VII. The loop is streaked rapidly back and forth covering the other half of the plate.

In applying our concepts to the study of variation, it is important to establish the precision with which quantitative data can be obtained. Making use of the definitions of rough and smooth cells, the percentage of smooth cells in rough colonies grown on standard agar at 37° C. for 24 hours was investigated by determining the percentage of smooth colonies on standard plates made from them. Two hundred and twenty-four plates were counted over a period of 60 consecutive days. The distribution of the values found is given in Fig. 3. The daily values are given in Fig. 4. The average value of the percentage of smooth colonies is 19·98. The average deviation is 1·71 and the deviation of the mean is

0·17. The distribution of the values obtained shows clearly that we are dealing with a biological constant.

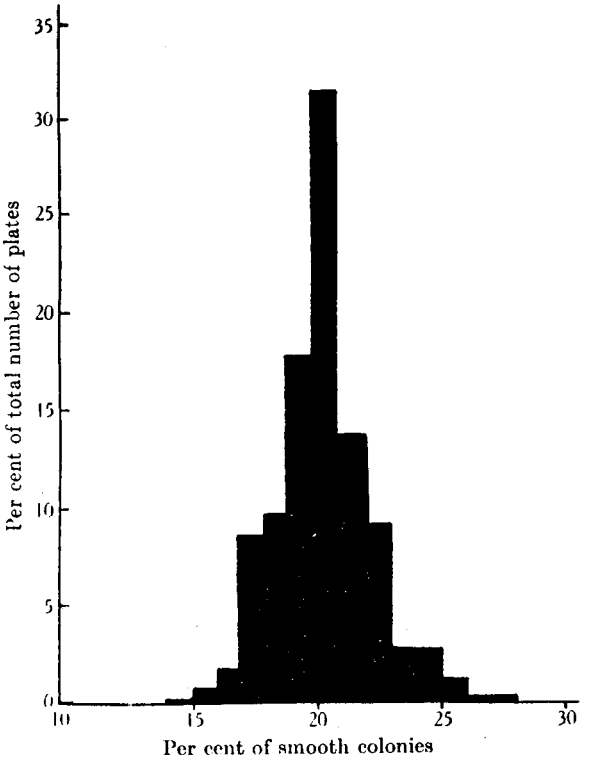

Fig. 3. Distribution of 224 differential counts on plates made from rough colonies grown at 37° C. for 24 hours. Percentage of total number of counts as a function of the percentage of smooth colonies found.

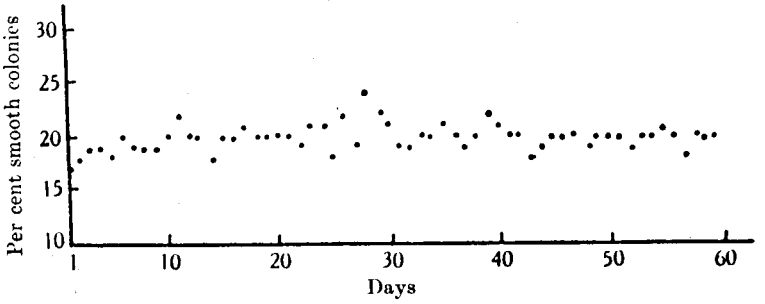

Fig. 4. Daily averages of percentage of smooth colonies found on plates made from rough colonies incubated at 37° C. for 24 hours. Sixty consecutive days.

To determine whether continued growth of the rough colony for an additional 24 hours affects the percentage of smooth cells, 48-hour-old

rough colonies were plated out. The colony counts made on these plates are similar to those on plates made from 24-hour-old colonies (Table I).

After it was established that a rough colony on standard agar at 37° C. contains approximately 20 per cent smooth cells at the end of 24 or 48 hours, the effect of lowering the temperature to 27° C. was investigated. It was found that, although at the end of 24 hours colonies grown at 27° C. were scarcely visible, at the end of 48 hours of growth the rough colonies were approximately the same size as those grown at 37° C. for 24 hours. Counts on sixty plates made from 48-hour-old colonies incubated at 27° C. gave an average of 37·3 per cent smooth colonies. The distribution of these counts is shown in Fig. 5. The average deviation is 2·3 per cent and the deviation of the mean 0·3 per cent.

TABLE I

	% smooth	Deviation
Colonies from plates inoculated with cultures grown at 37° C. and incubated 48 hours at 37° C., test plates at 37° C.	19	0·6
	20	0·4
	22	2·4
	20	0·4
	19	0·6
Two passages at 37° C. for 48 hours each, then a test plate at 37° C.	21	1·4
	18	1·6
	18	1·6
	18	1·6
	19	0·6
	Mean 19·6	1·07
		Deviation of mean 0·36

From a genetic standpoint it was important to determine whether the percentage of smooth cells in a rough colony depended in any way upon the previous history of the particular parent rough cell or entirely upon the environmental conditions of development of the colony itself.

A large series of passages was made in which plates were streaked from 37° C. colonies and incubated at 27° C., or streaked from 27° C. colonies and incubated at 37° C. In some cases several passages at 27° C. were followed by one or more passages at 37° C. in a large variety of combinations. A portion of this experiment is represented in Fig. 6. In all cases, plates streaked from cultures grown at 27° C. had the percentage of smooth colonies characteristic of that temperature, regardless of the temperature at which the test plate was grown. The counts shown in Fig. 5 were all made during the course of this experiment. Plates made from cultures grown at 37° C. had the percentage of smooth colonies characteristic for that temperature. In no case was more than one passage required to bring the composition of the culture to the value characteristic for the temperature at which it was grown.

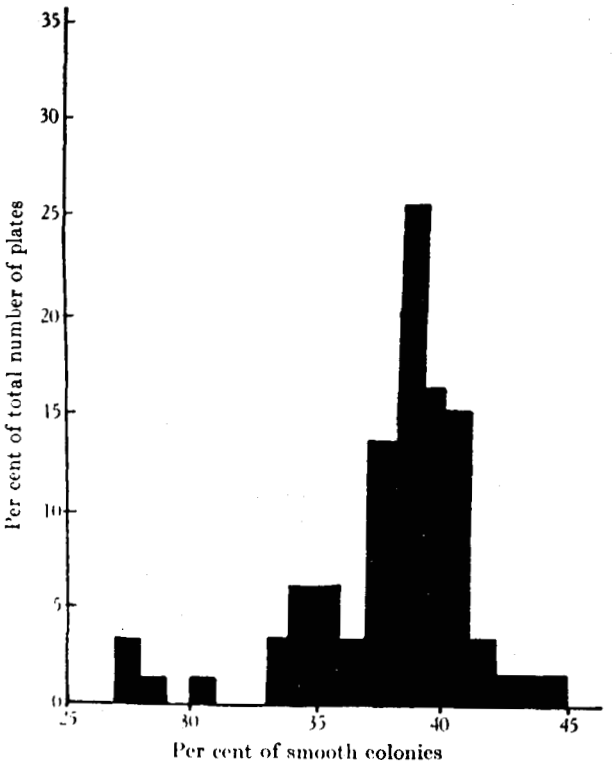

Fig. 5. Distribution of sixty differential counts on plate made from rough colonies incubated at 27° C. for 48 hours. Percentage of total number of counts as a function of the percentage of smooth colonies found.

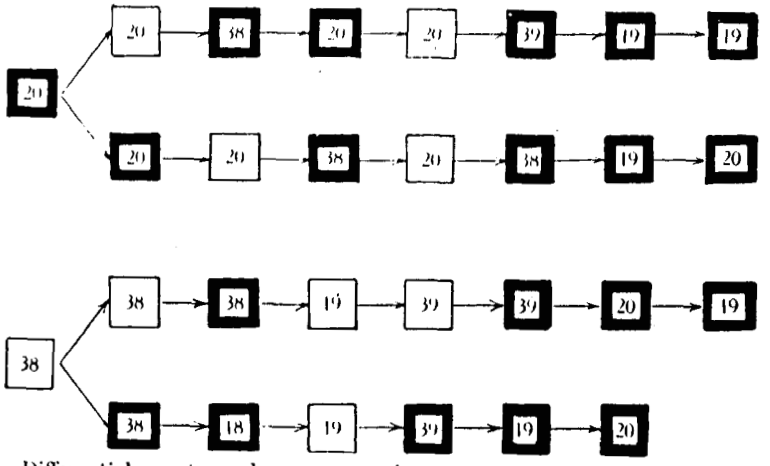

Fig. 6. Differential counts made on consecutive passages at 37° C. for 24 hours and at 27° C. for 48 hours. Numerals surrounded by a dark frame represent counts on plates grown at 37° C. Numerals surrounded by a light frame represent counts on plates grown at 27° C.

Since the percentage of smooth cells in any rough colony is the same as the percentage of smooth colonies in a plate made from it, the inevitable conclusion is that the percentage of smooth cells in a rough colony depends on the temperature at which that colony is grown, and not on the antecedent history of the parent cell.

This was demonstrated more spectacularly by the following experiment: five plates were streaked from 24-hour-old rough colonies grown at 37° C. and, instead of being allowed to develop in the incubator, were placed in a refrigerator for several months. At the end of this time no growth was discernible. When, however, these plates were transferred to the incubator at 37° C., the colonies developed, as usual, 20 per cent smooth and 80 per cent rough.

Discussion

We have demonstrated two characteristics of the rough cell. One is its ability to give rise to smooth cells, in addition to rough cells; and the other is its ability to produce a rough colony with a definite percentage of smooth cells, characteristic of the temperature of growth.

Since the smooth cells derived from rough cells continue to give rise to smooth cells only, we must consider that the change from rough to smooth is mutation. On the other hand, since the percentage of smooth cells in a colony is dependent entirely on the temperature at which it develops, and not upon the percentage of smooth cells in the colony from which the parent cell came, this change in rough cells, as determined from the properties of colonies grown from them, is not mutation but adaptation to change in environment.

Casual inspection of the percentage of smooth cells in a rough colony might suggest that we are dealing with whole number relationships (20 per cent is 1 to 4 and 37 per cent is almost 2 to 5). Since, however, smooth cells are derived from rough cells early in the development of the colony and continue to multiply, it cannot be considered that the percentage composition of the colony at any time is a measure of the percentage of smooth cells arising from rough cells. The kinetics of the exponential phase of the growth of a rough culture have been analysed previously (2) and shown to be in agreement with the idea that a constant percentage of the cells derived from rough cells are smooth cells. Further work in our laboratory on the analysis of the development of a rough culture has shown that large changes in the composition of the culture continue to take place after the culture has ceased to grow exponentially, when it is in the so-called stationary phase. Since the stationary phase

is analogous to a tissue, we believe that the kinetics of variation in this phase of bacterial growth will shed light on the general problem of cell variation in tissues.

SUMMARY

1. Macroscopic characteristics of bacterial cultures have been studied as a means of characterizing cells from which they are derived.

2. By single-cell micro-isolation, it has been demonstrated that the occurrence of smooth cells in rough colonies is due to mutation.

3. It has been shown that a rough colony grown at 37° C. has 20 per cent smooth cells and that a rough colony grown at 27° C. has 37 per cent smooth cells.

4. It has been shown that the percentage of smooth cells in a rough colony depends on the incubation temperature, and not on the history of the cell from which it is derived.

5. Further possibilities of this method of study have been suggested.

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